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Accepted Version

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Wei, X., Handoko, D. D., Pather, L., Methven, L. and Elmore, J. S. (2017) Evaluation of 2-acetyl-1-pyrroline in foods, with an emphasis on rice flavour. Food Chemistry, 232. pp. 531-544. ISSN 0308-8146 doi:
<https://doi.org/10.1016/j.foodchem.2017.04.005> Available at
<https://centaur.reading.ac.uk/69971/>

It is advisable to refer to the publisher's version if you intend to cite from the work. See [Guidance on citing](#).

To link to this article DOI: <http://dx.doi.org/10.1016/j.foodchem.2017.04.005>

Publisher: Elsevier

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1 Evaluation of 2-acetyl-1-pyrroline in foods, with an emphasis on rice
2 flavour

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ABSTRACT

The popcorn-like aroma compound 2-acetyl-1-pyrroline (2-AP) is a key contributor to the desirable aroma of fragrant rice and is also important in the aroma of other foods, such as pandan leaf, popcorn and Mediterranean sausage. It can be formed enzymatically in the rice grain as it grows and is also formed, as part of the Maillard reaction, when rice is heated. This review examines the formation of 2-AP in rice and other foods, particularly its formation during cooking, focusing on the importance of the Maillard reaction between reducing sugar breakdown products and 1-pyrroline derived from the amino acids proline and ornithine. The synthesis of 2-AP is discussed alongside the attempts that have been made to stabilise this relatively unstable compound. The analysis of 2-AP by instrumental techniques, particularly gas chromatography-mass spectrometry and gas chromatography-olfactometry, alongside the use of sensory studies, is also discussed.

Keywords: 2-acetyl-1-pyrroline, 2-AP, flavour, rice, pandan, popcorn, Maillard reaction, biosynthesis, analysis

26

27 **1. Introduction**

28 The IUPAC name of 2-acetyl-1-pyrroline (2-AP) is 1-(3,4-dihydro-2*H*-pyrrol-5-
29 yl)ethanone, its CAS number is 85213-22-5 and its FEMA (Flavor and Extract
30 Manufacturers Association) number is 4249. 2-AP was first identified in rice by
31 Buttery, Ling, and Juliano (1982), and is regarded as the most important aroma
32 compound in rice, especially fragrant rice (Buttery, Ling, Juliano, & Turnbaugh,
33 1983). In that study, 0.05 ppm 2-AP was described as popcorn-like and its odour
34 threshold in water was measured as 0.1 nL/L, while its odour threshold in air was
35 reported by Schieberle (1991) as 0.02 ng/L; this very low threshold makes it an
36 important contributor to a food's aroma when present. As well as rice, it is also a key
37 flavour compound in many cereal products, as well as some vegetable and animal
38 products (Adams & De Kimpe, 2006; Wakte, Zanan, Hinge, Khandagale, Nadaf, &
39 Henry, 2016).

40 Bioformation of 2-acetyl-1-pyrroline in both plants and microorganisms has been
41 studied and several types of bacteria are able to form this compound (see Part 3 of this
42 review). 2-Acetyl-1-pyrroline has also been shown to form in the Maillard reaction; it
43 can be formed from the reaction between proline and reducing sugars/sugar
44 degradation products upon heating (Schieberle, 1989).

45 Although there is a high commercial interest in 2-AP because of its desirable
46 sensory attributes, the instability of this compound is a significant problem for its
47 commercial application. Pure 2-AP will turn red and degrade within 10 minutes at

room temperature (Fang & Cadwallader, 2014), and there is significant short-term reduction of 2-AP concentration in food products, such as popcorn (Schieberle, 1995) and raw fragrant rice (Widjaja, Craske, & Wootton, 1996a).

The occurrence of 2-acetyl-1-pyrroline in food products, its bioformation and thermal formation, synthesis, stabilisation, analysis and sensory evaluation will be reviewed in this paper, with particular emphasis on the role of 2-AP in fragrant rice aroma.

2. Food sources of 2-acetyl-1-pyrroline

2.1. Rice

Non-fragrant rice (long and medium grain *indicas* and short grain *japonicas*), mainly grown in USA, Vietnam, Thailand and Australia, constitutes around 80% of the world rice trade (Singh, Singh, & Khush, 2000). Major producers of fragrant rice are India, Pakistan and Thailand. Most of the fragrant rice exported from India and Pakistan is basmati, while fragrant jasmine rice is a major export of Thailand (Singh et al., 2000). In 2010, Thailand was the biggest exporter of fragrant rice: 2.65 million tonnes of jasmine rice were exported, followed by India (1.80 million tonnes basmati) and Pakistan (1.05 million tonnes basmati) (Slayton & Muniroth, 2011).

The price of fragrant rice is much higher than that of non-fragrant rice. For example, high-quality fragrant basmati rice has a three times higher price than high quality non-fragrant rice. The commercial value of fragrant rice is higher than that of

non-fragrant rice, partly because fragrant rice varieties are relatively low yielding. Fragrant rice is less resistant to disease and insect pests and is prone to high shedding, leading to losses in yield (Berner & Hoff, 1986; Golam et al., 2011). It has been shown that higher quality grains with stronger aromas are generated in crops grown in drought and saline conditions (Yoshihashi, Nguyen, & Kabaki, 2004). These adverse conditions do not favour high yields.

2-AP is the key discriminator between fragrant and non-fragrant rice and many studies have focused on the concentration of 2-AP in different rice cultivars. 2-AP concentrations in different fragrant cultivars vary substantially (Table 1). For example, 2-AP was present in milled Fowler Gourmet Aromatic rice (a US-grown aromatic rice) at 999 $\mu\text{g/kg}$, while, in a set of five basmati samples, levels of 2-AP from 19 $\mu\text{g/kg}$ to 342 $\mu\text{g/kg}$ were measured (Bergman, Delgado, Bryant, Grimm, Cadwallader, & Webb, 2000).

Milled rice (commonly referred to as white rice) is obtained from the milling of brown rice to remove the outer bran layer. Whole rice grains are dehulled; then the dehulled (brown) rice is milled twice. Generally, 20–22% of the rice grain is hull, and another 8–10% is bran and embryo; therefore, the yield of milled rice is around 70% (Singh et al., 2000). As can be seen in Table 1, in most cases more 2-AP is present in brown rice compared to milled rice.

Caution should be applied when comparing data acquired by different authors. In some cases 2-AP was measured in uncooked rice (e.g., Hopfer, Jodari, Negre-Zakharov, Wylie, & Ebeler, 2016), while in other cases the rice was cooked before

analysis (e.g., Widjaja et al., 1996a; Widjaja, Craske, & Wootton, 1996b) and even during analysis (e.g., Buttery et al., 1983). The effect of sample preparation on 2-AP content in rice is covered in more detail in Part 7 of this review.

Soil and climate conditions during cropping can also influence 2-AP concentration in rice cultivars. During cultivation, a dry climate or sandy soil with low moisture retention can induce the fragrant rice cultivar Khao Dawk Mali 105 to produce more 2-AP (Yoshihashi et al., 2004). It appears that moisture during cultivation could be one of the most important factors affecting 2-AP formation when rice grows.

Due to the instability of 2-AP, drying and storage of rice can also influence the 2-AP content of the final product (Wongpornchai et al., 2004). The unstable nature of 2-AP will be discussed in detail in Part 6 of this review.

2.2. Pandan

2-AP is an important component of pandan leaf; the aroma of 2-AP is often described as pandan-like. Pandan plays an important role in south-east Asian cookery. The leaf of this plant is often boiled with rice to enhance flavour. When boiled with non-fragrant rice, it can provide the popcorn-like flavour associated with boiled fragrant rice, allowing cheap non-fragrant cultivars to possess similar aroma to higher value fragrant rice cultivars (Peter, 2006). The treatment of pandan leaf can affect 2-AP content. The fresh or slightly withered leaf is normally torn into strips, tied in a bunch and then boiled together with rice. The pandan leaves are removed from the rice after cooking.

The concentration of 2-AP in pandan leaves ranges from 40 ± 10 to 450 ± 10 $\mu\text{g/kg}$ (Yahya, Lu, Santos, Fryer, & Bakalis, 2010). Dried and ground pandan leaves were extracted in this study. However, those treatments disrupted the papillae structure in epidermal cells on the surface of the pandan leaves. 2-AP is contained in the papillae; therefore, a proportion of 2-AP is lost during drying and grinding.

2.3. Cereal products

2-Acetyl-1-pyrroline has also been detected in cooked cereal-based products. Wheat bread crusts contain around $75 \mu\text{g/kg}$ 2-AP compared to $1\text{--}4 \mu\text{g/kg}$ in sourdough processed rye bread (Schieberle & Grosch, 1987). Popcorn-like aroma compound 2-AP is, unsurprisingly, present in popcorn. However, in popcorn 2-acetyltetrahydropyridine and 2-propionyl-1-pyrroline also contribute roasty and popcorn-like flavour. The alkyl side chains of those compounds are short; only one or two carbon atoms length. In contrast, 2-butanoyl-1-pyrroline and 2-hexanoyl-1-pyrroline, compounds with similar structure but with longer alkyl side-chains, do not possess roasty or popcorn-like aroma (Schieberle, 1991).

2-AP was also identified in a cereal coffee brew at $8 \mu\text{g/L}$ and contributed intense popcorn-like odour attributes when analysed by gas chromatography-olfactometry (Majcher, Klensporf-Pawlik, Dziadas, & Jeleń, 2013). The cereal coffee was a roasted mixture of 40% barley, 25% rye, 25% chicory, and 10% sugar beet.

2.4. Other foods

2-AP has also been detected in non-cereal-based food. A high concentration of 2-

AP of up to 750 µg/kg was found on the surface of Mediterranean dried sausages, while values at the core were up to 100 µg/kg. *Penicillium nalgiovense*, the dominant mould species present, was shown to synthesise 2-AP during sausage processing (Stahnke, 2000). Using gas chromatography-olfactometry (GC-O), Blank, Devaud, Fay, Cerny, Steiner, and Zurbruggen (2001) identified 2-AP as a key contributor to the aromas of both Parma ham and Italian-type salami. They described the compound as it eluted from the GC column as having a ‘roasty’ aroma in the Parma ham and a ‘roasty, popcorn’ aroma in the salami.

2-AP was also isolated in Manuka honey at concentrations of 80–450 µg/kg. It was formed from methylglyoxal, which is responsible for the antibacterial activity in Manuka honey. Reaction of methylglyoxal with proline through the Strecker reaction can form 2-AP (Ruckriemen, Schwarzenbolz, Adam, & Henle, 2015). In addition, 2-AP was also isolated from two kinds of cooked edible fungus: huitlacoche and austern pilzen (Lizarraga-Guerra, Guth, & Lopez, 1997), but the compound was mistakenly identified as 2-acetyl-2-pyrroline (Adams & De Kimpe, 2006). The importance of 2-AP in mushroom (*Agaricus bisporus*) aroma increased significantly as a result of pan-frying (Grosshauser & Schieberle, 2013), its concentration rising from 0.4 to 5.3 µg/kg. Similarly, 2-AP was also detected in both raw and roasted hazelnuts; a significant increase of 2-AP concentration was observed, from trace levels (< 3 µg/kg) to 85 µg/kg, when hazelnuts were roasted (Kiefl, Pollner, & Schieberle, 2013).

2-AP may not always make a positive contribution to food aroma. An undesirable ‘mousy’ flavour in wetted raw pearl millet grits was attributed to 2-AP. Although 2-

AP concentration was not quantified in this study, it was implied that there was a higher concentration of 2-AP in millet than in rice, which was reflected in the difference in their odour quality (Seitz, Wright, Waniska, & Rooney, 1993).

2-AP has been identified and quantified in many food products. Table 2 shows those foods other than rice where 2-AP has been quantified. Even at a very low concentration, such as 3 µg/kg in milk chocolate (Liu, et al., 2015), this compound can still be considered a key odorant. In a recent review, a comprehensive list of food sources of 2-AP was provided, which included fruit and vegetables, fungi, cooked meat and fish, dairy and egg products (Wakte et al., 2016).

2.5. 2-AP as a flavouring

Several patents have suggested that 2-AP could be applied as a food flavouring. A food coating with a content of at least 40 ppb 2-AP, made from fragrant rice, was applied to increase popcorn odour in several products (Richard, 2001). In distilled alcoholic beverages, 0.2 to 200 ppb 2-AP contributed to a fragrant rice flavour (Asano et al., 2000). 2-AP was included in GRAS 22 (Smith et al., 2005) and the average and maximum levels for its addition to various food products have been summarised (Adams & De Kimpe, 2006).

3. Biological formation of 2-acetyl-1-pyrroline

3.1. Fragrant rice

It was originally thought that 2-AP was only produced during the cooking of rice

176 *via* the Maillard reaction (Buttery et al., 1982). However, further research has shown
177 that 2-AP is produced by the rice plant, and is detected in the majority of plant tissues
178 (Sakthivel, Sundaram, Rani, Balachandran, & Neeraja, 2009; Sood & Siddiq, 1978;
179 Yoshihashi, Huong, & Inatomi, 2002). It is now generally accepted that although
180 some 2-AP in rice is produced during cooking, 2-AP is predominantly biosynthesised
181 in rice.

182 Yoshihashi (2002) reported that 2-AP cannot be formed during the cooking of
183 fragrant rice (when heated with or without water at 90 °C for 8, 10, 12, 14 min, the
184 concentration of 2-AP showed a slight decrease), nor in postharvest processes like
185 drying and storage. It can only be formed in the aerial parts of plants during growing
186 in paddy fields. In a later paper by the same author, excised callus (cells covering a
187 plant wound) and seedlings were floated on labelled amino acid (200 ppm ¹⁵N-
188 glycine, ¹⁵N-L-proline or 1-¹³C-L-proline; pH 5.5) solutions. After incubation at 27 °C
189 in darkness for 8 hours, increasing 2-AP concentrations were detected. Results
190 showed clearly that the labelled derivative was only found in seedling and callus
191 incubated with ¹⁵N-L-proline. This result indicated that one of the precursors in 2-AP
192 biosynthesis could be proline but not glycine and that the nitrogen source of 2-AP is
193 proline. On the other hand, because no labelled derivative was found in the 1-¹³C-L-
194 proline sample, the acetyl group in 2-AP could not be provided by proline
195 (Yoshihashi, Huong, & Inatomi, 2002).

196 It appears that moisture during cultivation could be one of the most important
197 factors affecting 2-AP formation when rice grows. 2-AP concentrations in fragrant

rice Khao Dawk Mali 105 from the Tung Kula Rong Hai region in north-east Thailand, where there is a drought-prone climate with sandy soil, were much higher than in the same rice grown in other areas of Thailand. Rice samples planted in clay soil can retain moisture during growth, resulting in lower 2-AP concentrations than those grown in sandy soil (Yoshihashi et al., 2004). Numerous studies have shown that proline accumulation occurs in higher plants due to different environmental stresses, such as drought, high salinity, high light and UV irradiation, heavy metals, oxidative stress and in response to biotic stresses (Szabados & Saviouré, 2010). For example, Rhodes, Handa, and Bressan (1986) showed that proline will accumulate in water-deficient plant cells through the glutamate pathway. In this study, tomato cells adapted to water stress induced with polyethylene glycol (PEG); a tenfold increase of proline synthesis was observed in the water-stressed cells. This research and that of Yoshihashi et al. (2004) suggest that more 2-AP will be synthesised when rice is grown in a dry climate, due to increased accumulation of its precursor proline.

In addition, a cool climate and early harvest could increase 2-AP concentration in fragrant rice varieties. Between 1992 and 1994, three brown fragrant rice cultivars from Japan (Hieri, Miyakaori and Sari Queen) were harvested once a year and their 2-AP concentration was analysed. It was found that 2-AP was higher in rice crops exposed to low temperature (day 25 °C/ night 20 °C) than high (day 35 °C/night 30 °C) or moderate temperature (day 30 °C/night 25 °C). In addition, from results across three years, 2-AP concentrations of early harvest samples were higher than samples harvested at normal time (Itani, Tamaki, Hayata, Fushimi, & Hashizume,

2004).

In a recent study, a partial least squares model was built, based on planting and harvesting conditions, that could predict 2-AP concentrations in Thai Jasmine Pathumthani 1 rice (Funsueb, Krongchai, Mahatheeranont, & Kittiwachana, 2016). The status of the rice plants was recorded during cultivation and after harvest, such as number of tillers (grain-bearing branches), plant height, root length, number of grains per plant and grain weight. Nitrogen and sodium concentrations, rice yield, shoot dry weight and number of tillers per plant all had significant influences on 2-AP concentration.

There are two mechanisms proposed for the accumulation of 2-AP in mature grains. In the first 2-AP is synthesised in leaves and stem sheaths and transported to mature grains, while in the second proline translocates from leaves into grains and 2-AP synthesis occurs in grains. Hinge, Patil, and Nadaf (2016) showed maximum 2-AP concentrations in mature grains, with less proline in the grain at that time than at other developmental stages. These results suggested that the first mechanism was more likely in the fragrant rice cultivars they were studying (Ambemohar-157 and Basmati-370).

The gene *BADH2* encodes an enzyme, betaine aldehyde hydrogenase (BADH2) (Bradbury, Fitzgerald, Henry, Jin, & Waters, 2005), which catalyses the oxidation of 4-aminobutanal to 4-aminobutanoic acid (GABA). 4-Aminobutanal is a high affinity substrate for the BADH2 enzyme (Oishi & Ebina, 2005; Trossat, Rathinasabapathi, & Hanson, 1997). In solution 4-aminobutanal exists in equilibrium with its cyclic form,

1-pyrroline (Struve & Christophersen 2003), a 2-AP precursor. Hence the oxidation of 4-aminobutyraldehyde reduces the potential for 2-AP synthesis (Kovach, Calingacion, Fitzgerald, & McCouch, 2009). Bradbury et al. (2005) identified a mutated version of the *BADH2* gene as being responsible for determining fragrance in rice, which has since been confirmed (Arikrit et al., 2011; Fitzgerald, Waters, Brools, & Henry, 2010; Kovach et al., 2009; Siddiq, Vemireddy, & Nagaraju, 2012). The mutated *BADH2* gene incurs a deletion of eight base pairs in exon 7, leading to early gene termination and production of a truncated non-functional BADH2 enzyme (Bradbury et al., 2005). Non-fragrant rice cultivars contain the *BADH2* gene and hence a functional BADH2 enzyme; whereas fragrant cultivars have the mutated *BADH2* gene and so produce a non-functional enzyme (Bradbury, Gillies, Brusheet, Waters, & Henry, 2008). This non-functional enzyme will not be able to oxidise 4-aminobutyraldehyde, leading to a build-up of 1-pyrroline and hence increased 2-AP synthesis. Recent studies have shown that there are various other mutations in the *BADH2* gene that may also lead to increased 2-AP production, such as a deletion of seven base pairs in exon 2 (Amarawathi, Singh, Singh, Singh, Mohaoatra, & Sharma, 2008; He & Park, 2015). Similar biosynthetic pathways for the formation of 2-AP have been reported in soy beans (Arikrit et al., 2011) and sorghum (Zanan, Khandagale, Hinge, Elangovan, Henry, & Nadaf 2016).

Another biosynthetic pathway of 2-AP was proposed by Huang, Teng, Chang, Chuang, Ho, and Wu (2008) that did not involve BADH2. Higher levels of pyrroline-5-carboxylate synthase enzyme, and hence increased conversion of glutamate to 1-

pyrroline-5-carboxylate, occurred in fragrant cultivars, in comparison to non-fragrant cultivars. It was suggested that the 1-pyrroline-5-carboxylate undergoes a reaction with methylglyoxal, giving rise to 2-AP, either directly or *via* degradation to 1-pyrroline.

Although very different (a comparison can be seen in Figure 1), both pathways could require 1-pyrroline in order to produce 2-AP. 1-Pyrroline has been shown to be a limiting substrate of the biosynthesis of 2-AP in a recent study, where both fragrant and non-fragrant rice calli were incubated with 1-pyrroline. In both cases, a significant increase in 2-AP production was observed, proving 1-pyrroline to be a key intermediate of 2-AP biosynthesis (Poonlaphdecha et al., 2016).

3.2. Formation of 2-AP by microorganisms

Microorganisms could also play an important role in 2-AP formation. During cocoa bean fermentation, yeasts, lactic acid, acetic acid, and various spore-forming bacteria, such as *Bacillus cereus*, are involved in the flavour-forming reactions. Some *Bacillus cereus* strains produce popcorn-like notes and 2-AP was produced by several of these strains incubated on standard plate count agar at 35 °C; 30–75 µg/kg 2-AP was produced after 2 days. A series of ¹³C and ¹⁵N experiments showed that 2-AP could be formed from glucose as carbon source, and glutamic acid and proline as nitrogen sources, through *Bacillus cereus* metabolism (Romanczyk, McClelland, Post, & Aitken, 1995).

In Mediterranean dried sausages, which have a popcorn-like odour and are very

different from Northern European sausages, 2-AP is also regarded as the key aroma compound. The main difference between Northern European sausages and Mediterranean dried sausages is a coverage of mould on the latter. 2-AP concentration on the surface of Mediterranean dried sausages is much higher than at the core. Therefore, it was suggested that the mould on the surface of Mediterranean dried sausages is able to produce 2-AP. *Penicillium nalgiovense* was isolated from the sausage surface and it was the dominating mould species. When incubated with and without various supplements, it was found that *Penicillium nalgiovense* could only produce popcorn odour when the sausage was present (Stahnke, 2000).

2-AP, together with other *N*-heterocyclic compounds 2-ethyltetrahydropyridine and 6-acetyl-1,2,3,4-tetrahydropyridine, could cause mousy off-flavour in wine (Herderich, Costello, Grbin, & Henschke, 1995; Strauss & Heresztyn, 1984), through the action of lactic acid bacteria (LAB). *Lactobacillus hilgardii* DSM 20176 was incubated with a defined *N*-heterocycle assay medium, which included D-fructose, ethanol, L-lysine, L-ornithine and mineral salts. It was found that L-ornithine stimulated 2-AP formation and repressed 6-acetyl-1,2,3,4-tetrahydropyridine formation, while L-lysine had the opposite effect (Costello & Henschke, 2002). It had previously been suggested that D-fructose and ethanol could provide the acetyl side-chain for 2-AP and 6-acetyl-1,2,3,4-tetrahydropyridine (Strauss & Heresztyn, 1984). A possible mechanism of fermentable carbohydrate and amino acid, forming 2-AP and 6-acetyl-1,2,3,4-tetrahydropyridine through LAB fermentation was proposed by Costello and Henschke (2002). This pathway is shown in Figure 2. L-Lysine could

form the intermediate 1-piperidine *via* cadaverine pathways, with the enzymes L-lysine decarboxylase and cadaverine aminotransferase involved (Fothergill & Guest, 1977). Pathways from putrescine to succinate *via* 1-pyrroline in *P. fluorescens* and *E. coli* (Jacoby & Fredericks, 1959; Kim, 1964) have been reported. Putrescine is the decarboxylation product of ornithine (Fothergill & Guest, 1977); hence, 1-pyrroline could be formed through the putrescine pathway from L-ornithine. Due to the presence of carbohydrates, such as ethanol and glucose/fructose, acetyl-CoA accumulated through the heterolactic pathway and reacted with intermediates 1-pyrroline and 1-piperidine to form 2-AP and 6-acetyl-1,2,3,4-tetrahydropyridine, respectively.

Adams and De Kimpe (2007) reproduced the work of Romancyk et al. (1995) and suggested *B. cereus* formed 2-AP by enzymatic acetylation of 1-pyrroline. 1-Pyrroline was formed from the degradation of ornithine and proline, as proposed by Costello and Henschke (2002; see above and Figure 2).

4. Formation of 2-acetyl-1-pyrroline through the Maillard reaction

2-AP is not only present in raw food, like rice and pandan leaf, but is also formed in many cooked products. Therefore, the Maillard reaction is also an important route to 2-acetyl-1-pyrroline. Schieberle (1988) tested several model systems containing different amino acids, and showed that, when heated with reducing sugars, only proline, lysine and alanine could form 2-AP, with proline giving the highest yield. Two ¹³C-labelling experiments were designed: in the first experiment, 1-¹³C-proline

reacted with unlabelled glucose, and in the second experiment, unlabelled proline reacted with U- ^{13}C -glucose (all six carbon atoms are labelled with ^{13}C). Both experiments were carried out at 170 °C for 30 min. In both experiments labelled carbon was only found in the acetyl group of 2-AP and much more ^{13}C was detected in the second experiment, which indicated that glucose could provide the acetyl group in 2-AP formation (Schieberle, 1988).

Schieberle (1990) showed that both proline and ornithine could react with 2-oxopropanal to form 1-pyrroline, the most important intermediate in 2-AP formation, and there was a higher yield with ornithine than with proline. Figure 3a shows the formation of 1-pyrroline *via* Strecker degradation of ornithine; both ornithine and citrulline, another amino acid, can generate 4-aminobutanal. Schieberle (1995) also hypothesised a mechanism of 1-pyrroline formation from proline and 1-deoxyosone through Strecker degradation (Figure 3b). This reaction starts with the formation of an iminium ion. After decarboxylation and water elimination, 1-pyrroline can be generated from hydrolysis of the iminium ion.

Rewicki et al. (1993) reacted unlabelled proline with 1- ^{13}C -glucose, and noted the formation of a 1:1 mixture of unlabelled and labelled 2-AP. A proposed mechanism is shown in Figure 3c. Two isomers of 1-deoxy-2,3-glucosone form in a ratio of 1:1 from the labelled sugar. They are converted to the dihydro form of diacetylformoin, which reacts with 1-pyrroline, to form 2-acetylpyrrolidine, which then oxidises to 1:1 labelled and non-labelled 2-AP (Rewicki et al., 1993). The 1:1 ^{13}C label was due to the 1:1 ratio of the reducing sugar fragments. The ^{13}C from labelled proline did not

exist in the final 2-AP product; supporting the theory that 2-AP is formed by acylation of 1-pyrroline by a two-carbon sugar fragment. Hofmann and Schieberle (1998a) also reported that 2-acetylpyrrolidine could oxidise to 2-AP and proposed that 1-pyrroline and 2-oxopropanal formed 2-acetylpyrrolidine *via* a number of steps. 2-Acetylpyrrolidine was then readily oxidised to 2-AP.

Phosphate ion could significantly increase the yield of 2-AP, through increased formation of 2-oxopropanal *via* 1,3-dihydroxyacetone phosphate (Schieberle, 1989). If malonate buffer replaced phosphate buffer, there was a one-third reduction of 2-AP formation (Schieberle, 1995). Blank, Devaud, Matthey-Doret, and Robert (2003) examined the effect of pH and heating time on the formation of various Maillard-derived compounds in two phosphate-buffered model systems: one an equimolar mixture of proline and glucose, the other the Amadori compound fructose-proline. 2-AP yield was similar in both systems across all treatments and was shown to increase with increasing pH and heating time, when samples were refluxed for 1, 2 and 4 hours at pH 6, 7 and 8.

From the above hypothesised mechanisms of 2-AP thermal formation, it is agreed that 2-AP is formed through an acylation of 1-pyrroline. Certain amino acids, i.e., proline, ornithine and citrulline, reacting with 2-oxopropanal from reducing sugar fragmentation, are the most important intermediates of 2-AP formation during the Maillard reaction (Adams & De Kimpe, 2006).

5. Synthesis of 2-acetyl-1-pyrroline

The first synthesis of 2-AP was reported by Buttery et al. (1983) and is shown in Figure 4a. This method is based on an earlier synthesis of a six-membered ring compound 2-acetyl-1,4,5,6-tetrahydropyridine (Buchi & Wuest, 1971). However, the yield of 2-AP from this reaction was only around 10%.

The first large-scale method for 2-AP synthesis was developed in 1993. Methylproline is oxidised to 2-(methoxycarbonyl)-1-pyrroline, which then reacts with methylmagnesium iodide in a Grignard reaction. However, this is not a completed reaction with a 45–83% yield and 8–39% starting material in the final product (De Kimpe, Stevens, & Keppens, 1993). Methylolithium in ether also converted 2-(methoxycarbonyl)-1-pyrroline into a mixture of 2-acetyl-1-pyrroline (47%) and a side-product, 2-(1-hydroxy-1-methylethyl)-1-pyrroline (32%) (Fig. 4b). To prevent formation of this side-product, which was also formed in the methylmagnesium iodide reaction, a cyanide functional group can replace the ester (Figure 4c). In this modified method, the reaction started with oxidation of pyrrolidine to tripyrroline. The tripyrroline was hydrocyanated into 2-cyanopyrrolidine, which can form 2-cyano-1-pyrroline through the Grignard reaction. 2-Cyano-1-pyrroline can form 2-AP with a yield of 60% when treated with methylmagnesium iodide (De Kimpe et al., 1993).

Over subsequent years, other synthesis methods focused on the stabilisation of 2-AP during the reaction, by using protected carbonyl groups, e.g., Duby and Huynh-Ba (1993), or amino groups, e.g., De Kimpe and Keppens (1996). De Kimpe and Keppens (1996) used diacetyl as a starting material to generate an α -diimine, which then reacted with a stabase derivative to form the 1-pyrroline ring structure (Fig. 4d).

Another synthesis method applied the high substrate selectivity of immobilised penicillin G acylase (PGA) as the catalyst in the last reaction step (Favino, Fronza, Fuganti, Fuganti, Grasselli, & Mele, 1996). 1-Aminohex-4-yne reacted with phenylacetyl chloride, and then ozone oxidised the product to form 1-[*N*-(phenylacetyl)amino]-4,5-dioxohexane, which when treated with PGA could form 2-AP spontaneously, as shown in Figure 4e. An 80% yield of 2-AP could be achieved using this method.

A four-step synthesis was reported by Hofmann and Schieberle (1998b) starting from *N*-*tert*-butoxycarbonyl-protected proline, while 2-pyrrolidinone was selected as a raw material for 2-AP synthesis by Harrison and Dake (2005). Another ‘popcorn’ compound, 2-acetyl-1,4,5,6-tetrahydropyridine, was a by-product of this latter method. A three-step synthesis was reported by Fuganti, Gatti, and Serra (2007), starting from the reaction of *N*-Boc-pyrrolidinone with ethylmagnesium bromide. The yield of 2-AP was only 20–30% but with 98% purity. Maraval et al. (2010) formed *N*,5-diacetylpyrrolidin-2-one from L-glutamic acid and acetic anhydride with 78% yield. Sodium carbonate was used for deacetylation to form 5-acetylpyrrolidin-2-one; then lithium aluminium hydride was used for reduction to form 2-(1-hydroxyethyl)-pyrrolidine, which was oxidised by silver carbonate to 2-AP. The overall yield of 5-acetylpyrrolidin-2-one formation was 37% but the yield of 2-AP was not reported.

A recent publication reported the synthesis of three major popcorn-like Maillard aroma compounds, 2-acetyl-1-pyrroline, 2-acetyl-1,4,5,6-tetrahydropyridine and 2-acetyl-5,6-dihydro-4*H*-1,3-thiazine (Deblender, Van Aeken, Adams, De Kimpe, &

Abbaspour Tehrani, 2015). The authors noted that existing synthetic procedures for these compounds suffered a number of problems, including extensiveness of some reaction pathways, and the use of costly and/or harmful reagents. The 2-AP synthesis they proposed started from *N*-Boc-proline to give a final yield of 2 AP of 28%, *via* a four-step reaction (Fig. 4f). The authors considered this method to be relatively straightforward, as the starting materials were readily available, only one general procedure was involved and the vinyl ether intermediate prepared was a stable precursor, which could be readily converted to 2-AP.

Although numerous procedures have been published for the synthesis of 2-AP, these methods all require an experienced organic chemist to make them work. Synthesis of 2-AP is difficult, due to the unstable nature of this compound, which degrades very rapidly upon standing (see Part 6). This is reflected in the high price of commercial 2-AP and the small number of 2-AP suppliers.

6. Stability and stabilisation of 2-acetyl-1-pyrroline

Stability is very important for a flavour compound in food products. Unfortunately, 2-acetyl-1-pyrroline has limited stability. Pure 2-AP will turn red and degrade within 10 min at room temperature (Fang & Cadwallader, 2014). This instability of 2-AP was noticed when it was first identified by Buttery et al. (1982), and this instability was assumed to be due to polymerisation. Loss of 2-AP in stored foods could be due to complexation, decomposition, diffusion to the environment and

generation of other compounds (Adams & De Kimpe, 2006).

An experiment was designed to investigate the effect of storage on 2-AP levels in rice (Widjaja et al., 1996a). In this experiment, using the fragrant cultivar YRF9, paddy (rice with husk and rice bran), brown (rice without husk but with rice bran) and white (rice without husk and rice bran) rice samples were stored under two conditions: atmospheric pressure and reduced pressure, at 84% RH and 30 °C. After three months' storage, 2-AP level was reduced by 40–50% in all cases. Another study aimed to compare the effect of different drying methods and storage time on 2-AP reduction in fragrant rice (Wongpornchai et al., 2004). Six different drying methods (sun drying, 30 °C modified air, 40 °C modified air, 40 °C air, 50 °C air and 70 °C air) were applied to fresh paddy rice, to reduce moisture content from 28% to below 14%, and then the rice was stored in gunnysacks at 20–35 °C. 2-AP concentration in 10 months stored rice was only 25% of freshly dried rice and it was shown in a concentration–storage time curve that a significant decrease occurred at the beginning of storage. The sun-dried sample retained less 2-AP than the other drying methods; this could be due to the longer drying time. Sun drying took 54 hours in this study, while the average time for the other drying methods was 10 hours. Although the authors did not provide details of the modified air used, drying with this kind of air maintained 2-AP in rice better than normal hot air drying, while lower air temperatures also resulted in less 2-AP loss (Wongpornchai et al., 2004).

In addition to rice, 2-AP decreases in other food products during storage. Hot air popped popcorn was sealed in commercial polyethylene food bags and stored in the

dark at room temperature. After two days, the 2-AP level reduced by 20% and after seven days storage, it reduced by 75% (Schieberle, 1995).

Therefore, it is important to develop a stabilisation method to defer 2-AP breakdown. Encapsulation is a popular technique to protect unstable volatile compounds for commercial processing, and several studies have applied this technique. Encapsulation of 2-AP by β -cyclodextrin (Duby & Huynh-Ba, 1996) showed some success. When stored at room temperature (20 °C), 99% 2-AP decomposed after 110 days' storage, when the 2-AP load of the β -cyclodextrin was 1%. However, if the storage temperature decreased, encapsulation performed better, with 10% losses at 4°C and no losses at -20 °C. If the loading of cyclodextrin was increased to 10%, the stability of the 2-AP was reduced.

Apintanapong and Noomhorm (2003) extracted 2-AP from pandan leaves and examined its stability at 30 ppm in acidic and basic solution at room temperature. They also microencapsulated 2-AP in various maltodextrin and gum acacia mixtures. In basic solution, 2-AP was reduced by 63% after 7 days, and in acidic solution by 30% after 35 days. When 2-AP was microencapsulated with 70:30 gum acacia:maltodextrin, only 28% of the encapsulated 2-AP was lost after 72 days at room temperature. Gum acacia and/or starch mixed materials were used in a patented form by Srinivas, Sulochanamma, Raghavan, and Gurudutt (2006), to form a stable 2-AP powder using spray drying, but the stability of this powder was not reported.

Fang and Cadwallader (2014) recently reported a novel stabilisation method, using zinc ions to solve 2-AP powder storage problems. Anhydrous 2-AP and ZnI_2 were

added into diethyl ether to form a yellowish precipitate, which was the desired 2-AP-ZnI₂ complex. Excess ZnI₂ and other impurities were removed through dissolving the complex in anhydrous diethyl ether. The complex compound was obtained as a powder after drying through nitrogen evaporation. Other 2-AP-zinc halide complexes could be obtained in the same way. When stored at 25 °C, there was only 6% loss of 2-AP from a 2-AP-ZnI₂ complex (2-AP content = 14.4%) after 3 months, and 3% reduction of 2-AP after 3 months when a 12.5% 2-AP content complex was stored at – 20 °C. It was found that compared with ZnI₂(2-acetyl-1-pyrroline)_n, which had a yield of 62%, complexes of ZnBr₂(2-acetyl-1-pyrroline)_n and ZnCl₂(2-acetyl-1-pyrroline)_n had better yields of 96% and 86%, respectively. A ZnCl₂-2-AP complex would be the preferred food agent because ZnCl₂ has been approved for food use (CFR – Code of Federal Regulations Title 21; April 1st, 2016). This method can also applied to similar volatile compounds, such as 2-propionyl-1-pyrroline, 2-acetyl-1,4,5,6-tetrahydropyridine, 2-acetyl-2-thiazoline, 2-acetylthiazole, 2-acetylpyrazine and 2-acetylpyridine. Although this is an effective technique for 2-AP stabilisation compared with others, this high yield was only confirmed in the dry powdered complex. It may be reduced by moisture, temperature and other conditions when applied in food. Therefore, it may be necessary to combine this technique with an encapsulation technique to protect the 2-AP-zinc halide complex in a changeable food environment (Fang & Cadwallader, 2014).

7. Extraction and instrumental analysis of 2-acetyl-1-pyrroline

7.1 Solvent-based extraction techniques

Simultaneous distillation extraction (SDE) was used as the extraction method when 2-AP was first discovered by Buttery et al. (1982). SDE was widely used in the 1980s and 1990s for volatile compound extraction (Chi, Yeung, & MacLeod, 1981). The sample is heated in water to produce steam and the steam transfers volatile material to a boiling non-polar solvent, which condenses to give an aroma extract (Likens & Nickerson, 1964). However, this vigorous heating process may cause volatile compound formation or breakdown. Buttery et al. (1983) reported that the boiling conditions used in this extraction may decompose 2-AP in rice and cause a lower concentration than in raw samples.

In Buttery's study, 500 g rice were extracted with 6 L water in a Likens-Nickerson type extraction equipment (Likens & Nickerson, 1964); diethyl ether was used as solvent and the isolation process was carried out at atmospheric pressure for 2 hours. After concentration, the solvent extract was dissolved in hexane and then extracted with 3 N hydrochloric acid and then ether. The ether extract was then concentrated to a small amount for analysis. For subsequent quantitative measurements (Buttery, Ling, & Mon, 1986), an internal standard (5 mL of 30 ppm 2,4,6-trimethylpyridine (collidine) solution) was added to the rice before extraction. Buttery's group published a number of papers on 2-AP in fragrant rice. They detected 2-AP in 10 different varieties of cooked rice (both milled and brown) using SDE and found that the concentrations of 2-AP in brown rice were much higher than in white rice (Buttery et al., 1983).

Likens-Nickerson extraction was continuously developed and used in the following decade for 2-AP extraction in rice, pandan leaf and other food samples. Addition of magnesium sulfate (MgSO_4) during rice SDE inhibited starch gelatinisation, water absorption, swelling of rice and foaming of the mixture during distillation (Widjaja et al., 1996a & b). Dichloromethane (DCM) has also been used as the extraction solvent in SDE of 2-AP (Nadaf, Krishnan, & Wakte, 2006).

Rice was boiled before extraction in some studies and in others the rice was boiled during SDE. For example, when fresh and stored brown and white fragrant YRF9 rice were compared, samples were boiled during the SDE process (Widjaja et al., 1996a & b). When white Italian Line B5-3 and basmati, two fragrant rice species, were compared, they were also boiled during SDE. A 4-fold higher 2-AP concentration was found in the Italian variety than in basmati (Tava & Bocchi, 1999). Several different varieties of brown fragrant rice (Malagkit Sungsong, 370 basmati, Khashkani and Indica) were boiled for 25 min in tap water before SDE analysis. 2-AP was found in all four species but the concentration in Indica was much lower than in the others (Jezussek, Juliano, & Schieberle, 2002). 2-AP was also detected in five boiled fragrant rice cultivars; four of them were white rice (Hyangmibyeo 1, Hyangmibyeo 2, Royal, Golden Elephant) and one a Korean black rice called Goemjeongssal. Boiled non-fragrant rice Jeongilpum also contained 2-AP. Those six cultivars were boiled 30 min with distilled water (Yang, Shewfelt, Lee, & Kays, 2008). Three white fragrant cultivars (Aychade, Fidji, and Giano) and one white non-fragrant cultivar (Ruille) were boiled for 20 min. 2-AP was found in all four cultivars, but the concentration in

Ruille was too low to quantify; it was lower than 2 µg/kg while the concentrations in the other fragrant cultivars were 150–300 µg/kg (Maraval et al., 2008).

When using SDE at atmospheric pressure, sample and water mixture are boiled. Therefore, this kind of extraction technique cannot be used to study uncooked foods. Buttery et al., when first identifying 2-AP, used simultaneous distillation/extraction under vacuum (V-SDE) to study rice aroma. However, compared to SDE at atmospheric pressure (A-SDE), V-SDE showed a low efficiency of extraction (Buttery et al., 1983). Levels of 2-AP in rice extracted by A-SDE were 10 times higher than in rice that was cooked and then extracted with V-SDE. The authors suggested that most of the 2-AP may be lost during cooking. Therefore, compared with A-SDE, where rice is cooked during the isolation process, less 2-AP is present in the already cooked sample in V-SDE. In addition, 2-AP may be generated during cooking, which can also cause the significant difference in concentrations obtained between V-SDE and A-SDE.

Another solvent-based extraction technique, solvent-assisted flavour evaporation (SAFE), first introduced in 1999 (Engel, Bahr, & Schieberle, 1999), is a useful technique for 2-AP extraction. The volatile compounds in a solvent extract, usually in diethyl ether or dichloromethane, are removed from non-volatile material using high-vacuum distillation. The procedure takes place at around 30 °C, keeping sample decomposition to a minimum. When using 1:1 diethyl ether:dichloromethane as the solvent, 2-AP was isolated from cereal coffee brew (Majcher et al., 2013) and this compound was also isolated from hazelnut when using diethyl ether as solvent (Kiefl

et al., 2013).

Solid-phase extraction (SPE) can also be applied for 2-AP extraction. Several commercial SPE cartridges (Strata™ X from Phenomenex, LiChrolut® EN from Merck Millipore and Isolute® ENV+ from Biotage) have been successfully used for volatile compound extraction (Du & Qian, 2008; Metafa & Economou, 2013), particularly for isolation of relatively polar aroma compounds, such as 2-AP. An advantage of SPE is that no heating is applied when using this technique, which is the same as SAFE, but SPE is much easier to perform than SAFE. 2-AP was generated during high-temperature cooking of fragrant rice (180 °C for 20 min in an open system), using SPE, followed by GC-MS (Handoko, 2014). This result suggests that there may be a component of fragrant rice that is formed enzymatically, which can be converted to 2-AP by the application of higher temperatures. Although there was no 2-AP detected in Ciherang rice (a non-fragrant rice) heated under the same conditions, a sensory panel perceived popcorn-like odour (Handoko, 2014), suggesting that compounds besides 2-AP that could cause popcorn-like odour in rice heated at 180 °C.

7.2 Headspace techniques

Dynamic headspace extraction using an adsorbent polymer such as Tenax can also be used in 2-AP extraction, Buttery, Turnbaugh, and Ling (1988) used this technique to analyse the volatile compounds in cooked rice. Seventeen volatile compounds include 2-AP were identified through this method. Around 0.6 µg/kg 2-AP were found in white Californian long-grain rice (a kind of non-fragrant rice) boiled in water for 20

min before Tenax trapping (Buttery et al., 1988). Around 30 odorants were identified in cooked rice using the same technique (Yang et al., 2008).

Headspace solid-phase microextraction (HS-SPME) is now the most widely used extraction method for 2-AP. As with all extraction techniques, increased extraction time or higher temperatures could result in better release of 2-AP from the sample. However, the reported instability of 2-AP may cause its loss during isolation at higher temperatures, while at lower temperatures enzymatic changes may occur during this extraction. These conflicting reactions make 2-AP quantification difficult.

One large study used manual SPME to analyse 91 different uncooked cultivars, including 77 non-basmati fragrant cultivars, 9 basmati fragrant cultivars and 5 non-fragrant cultivars. A 1-cm divinylbenzene/Carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fibre was used in this experiment. Samples were extracted for 15 min at 80 °C after a 30-min equilibration period. 2-AP was detected in some non-fragrant cultivars but its average concentration was around 10-fold higher in basmati fragrant cultivars and around 20-fold higher in selected non-basmati fragrant cultivars (Mathure, Wakte, Jawali, & Nadaf, 2011; Mathure, Jawali, Thengane, & Nadaf, 2014). Bryant and McClung (2011) used an automated SPME system to compare seven uncooked fragrant and two uncooked non-fragrant rice samples extracted with a 1-cm DVB/CAR/PDMS SPME fibre at 80 °C for 18 min after a 5-min equilibration period. 2-AP was only found in the fragrant rice samples.

A recent study measured 2-AP in 48 fragrant rice samples using manual SPME, followed by GC-MS/MS. The ion transition from m/z 111 to m/z 82 was used to

quantify 2-AP. Optimised conditions were 10 minutes extraction at 40 °C after a 5-min thermostating period. The technique was sensitive enough both to quantify 2-AP below its odour threshold concentration (Buttery et al., 1983) and to obtain successful 2-AP measurements using a single grain of rice (Hopfer et al., 2016). Although DVB/CAR/PDMS fibres performed better than DVB/PDMS fibres, the latter were preferred, because carry-over (the presence of volatile material from the fibre in a subsequent blank analysis) observed when using the DVB/CAR/PDMS fibre was not observed with the DVB/PDMS fibre. A limit of quantification of 103 ng per kg was reported for 2-AP in this work.

7.3 Gas chromatography-mass spectrometry

Gas chromatography-mass spectrometry (GC-MS) is the most common technique used for volatile compound analysis. Column choice in GC-MS analysis of 2-AP is very important. A polar phase (e.g., Carbowax) is normally chosen; 2-AP is a relatively polar compound and its peak shape is more symmetrical and sharper on a polar column. The use of base-deactivated phases is recommended for basic compounds that may possess poor peak shape under normal GC conditions (De Zeeuw, Stricek, & Stidsen, 2011). A base-deactivated column may also be useful for quantifying 1-pyrroline (Poonlaphdecha et al., 2016). Because of the instability of 2-AP, Buttery et al. (1986) recommended a relatively low injector temperature of 150-170 °C to minimise its decomposition. Base-deactivated injection port liners may also have a protective role (De Zeeuw et al., 2011).

When quantifying 2-AP using GC-MS, separation of 2-AP from other compounds

is a common challenge. This interference problem was first reported by Paule and Powers (1989) when using a packed column coated with 10% Carbowax 20M on Chromosorb® W; 1-hexanol eluted very close to 2-AP and could interfere in its quantification. The mass spectrum of 6-methyl-5-hepten-2-one contains all the major ions present in the mass spectrum of 2-AP (m/z 43, 41, 111, 83, 68, 69); these two compounds often co-elute in fragrant rice extracts run on polar columns, affecting the quantification of 2-AP, especially when the concentration of 2-AP is similar to or lower than that of 6-methyl-5-hepten-2-one. A long isothermal stage of 65 °C for 70 min at the start of the GC run was reported by Tanchotikul and Hsieh (1991) when performing sample analysis by GC-MS using a 60-m length Supelcowax® 10 (Supelco, Bellefonte, PA) column. A similar method was reported by Seitz et al. (1993), to obtain better separation of 2-AP and 6-methyl-5-hepten-2-one. They used a shorter, 30 m Supelcowax 10 column in this analysis at an initial temperature of 60 °C for 15 min.

Although electron ionisation (EI) is the usual ionisation mode used for GC-MS, chemical ionisation (CI) is an option. CI is a softer ionisation technique, which can reduce interference during MS analysis compared with EI, and hence could result in increased signal-to-noise ratio for compounds of interest. In a study on bread flavour by Schieberle and Grosch (1987), 2-AP was analysed in CI mode using isobutane as reagent gas. Compared with EI mode in GC-MS analysis, Maraval et al. (2010) reported that positive ion CI mode could be better for 2-AP quantification in rice, especially when MS-MS was applied for analysis. The EI mass spectrum of 2-AP

possesses few defining peaks: a characteristic ion at m/z 83 and a less intense molecular ion at m/z 111. However, in PCI mode, using acetonitrile as the reagent gas, only an intense pseudomolecular ion at m/z 112 was observed. Under MS-MS conditions, m/z 112 ion yielded a fragment ion at m/z 70 and this transformation was used for 2-AP quantification, with a low limit of quantification of 0.4 $\mu\text{g/kg}$.

7.4 Quantification of 2-acetyl-1-pyrroline

When Buttery et al. (1983) first quantified 2-AP in rice they measured peak areas by flame ionisation detector and performed an approximate quantification, in order to determine the relative amounts of 2-AP in the 16 types of rice that they analysed. In subsequent work they used collidine (2,4,6-trimethylpyridine) as an internal standard, adding it in solution to the rice/water mixture prior to extraction. Collidine was chosen because it has similar physicochemical properties to 2-AP (basic, similar water solubility, similar volatility), is stable, has a GC retention time similar to 2-AP on a wax column and is commercially available (Buttery et al., 1986). Known amounts of 2-AP were added to the rice prior to extraction alongside a fixed amount of collidine, in order to provide a calibration curve for quantification. Collidine was subsequently used as the internal standard in a number of papers where 2-AP was quantified in rice (Tanchotikul & Hsieh, 1991; Widjaja, et al. 1996a & b; Tava & Bocchi, 1999; Bergman et al., 2000).

Stable isotope dilution assays (SIDA) are now widely used in flavour science. An isotopomer of the compound of interest is added to the sample under study, in order to permit accurate quantification of the compound of interest. The quantification of 2-AP

by SIDA was carried out for the first time by Schieberle and Grosch (1987). They prepared a 2-AP analogue, which was partially deuterated in the heterocyclic ring, giving a product with a range of molecular masses from 113 to 116. They then used the deuterated isotopomer to quantify 2-AP in wheat and rye bread.

SIDA was used to measure 2-AP in rice for the first time by Yoshihashi et al. (2004). Instead of deuteration, a ^{13}C atom was introduced in the methyl position of the acetyl side-chain giving an isotopomer with a mass of 112. Naturally-occurring 2-AP has an M+1 ion with a mass of 112, which has 7% of the intensity of its molecular ion. It is not clear if this was taken into account by the authors in their calculations. This issue was highlighted by Maraval et al. (2010), who used SPME with deuterated 2-AP to quantify 2-AP in rice. Unlike Schieberle and Grosch (1987), the deuteration was defined. Deuterium-hydrogen exchange can occur in aqueous solution and to reduce the chances of this happening the authors replaced both hydrogen atoms at the 5-position of the heterocyclic ring with deuterium. In order to provide a calibration curve for quantification, ground leaves from a non-scented rice cultivar were spiked with nine different amounts of 2-AP in solution.

The key reason for performing SIDA is that several steps of enrichment of the compounds can be performed without losses in accuracy, provided that the initial ratio between the compound and its labelled analogue remains unchanged during the entire procedure (Schieberle & Grosch, 1987). As the compound of interest and its isotopomer should have the same physicochemical properties, SIDA provides a degree of confidence that is lacking when other internal standards are used. The incompletely

deuterated 2-AP synthesised by Schieberle and Grosch is now available commercially from AromaLab AG (Planegg, Germany) and has been used to quantify 2-AP in rice (Hopfer et al., 2016).

8. Sensory evaluation of 2-acetyl-1-pyrroline

2-AP is described as a popcorn-like odour compound and it has a very low odour threshold. When Buttery et al. (1983) first identified this compound, they ranked the amount of popcorn-like odour in different rice varieties. Malagkit Sungsong, a kind of Philippine fragrant rice, had the greatest popcorn aroma and Texas Long Grain was determined as the rice with the least popcorn aroma. The most famous fragrant rice, basmati, was ranked in the middle of this list. When the Malagkit Sungsong was compared with Calrose (a non-fragrant rice), it was easy to distinguish them. However, when a 2-AP solution was added to the Calrose rice, they became much more difficult to tell apart. It was clear that the popcorn aroma of 2-AP is a key component of rice flavour.

Lexicons of aroma and flavour of rice are being continuously developed by researchers (Goodwin et al., 1996; Piggott, Morrison, & Clyne, 1991; Yau & Liu, 1999). When comparing these studies, some descriptors are similar, but some are different; it is difficult to estimate which research has an intact lexicon and which needs more development. The choice of descriptors depends on the culture and familiarity with the sample of the panellists in each study (Paule & Powers, 1989). A study aiming to build an intact lexicon tested 36 different varieties of rice, which were

mainly jasmine and basmati rice samples from different regions, but also included many other fragrant and non-fragrant rice species (Limpawattana & Shewfelt, 2010). Twenty-four attributes were listed by 8 trained panellists, of which 6 did not vary across the 36 varieties. The 18 attributes finally used in this study were ‘popcorn’, ‘starchy’, ‘woody’, ‘cooked-grain’, ‘grain’, ‘sulfury’, ‘corn’, ‘nutty’, ‘floral’, ‘dairy’, ‘hay-like’, ‘barny’, ‘buttery’, ‘green’, ‘rancid’, ‘waxy’, and ‘earthy’. A standard and intensity of standard for each attribute was also defined. Of the 18 significant attributes, ‘popcorn’, which was mainly attributed to 2-AP, was positively correlated with ‘buttery’ and ‘corn’ and negatively correlated with ‘earthy’ and ‘smoky’.

In an earlier study from Limpawattana’s group, sensory profiling was conducted on 13 varieties of rice by using trained panels, and aroma-active compounds were analysed by GC-olfactometry (GC-O) and GC-MS (Limpawattana, Yang, Kays, & Shewfelt, 2008). In this study, a predictive model was built for correlation analysis of attributes and volatile compounds. Unexpectedly, ‘popcorn’ in this model was negatively correlated with guaiacol and (*E,E*)-2,4-decadienal, while 2-AP was not present in this model as a ‘popcorn’ descriptor. Guaiacol and (*E,E*)-2,4-decadienal contributed smoky and fatty attributes, respectively. The authors suggested that the thermal process of reference standard preparation may have influenced the ‘popcorn’ descriptor analysis. In addition, these authors suggested that the contribution of 2-AP to popcorn-like odour was always overemphasised relative to many other compounds which also contribute to this aroma in fragrant rice.

Three types of fragrant rice (jasmine, basmati, and Jasmati) were studied in a

recent paper, to compare their main aroma active compounds using GC-O and GC-MS (Mahattanatawee & Rouseff, 2014). Hexanal, octanal, 2-AP, (*E,E*)-2,4-nonadienal, (*E*)-2-nonenal, 4-vinyl-2-methoxyphenol and indole were identified as the aroma-active compounds common to all three species. Across all three types of rice, 30 compounds were identified as aroma-active compounds and were described by 8 attributes. Jasmati contained 35% less ‘roasty/nutty’ total aroma intensity than jasmine and basmati, while ‘medicine’ flavour was not detected in jasmine rice. Jasmine contained 35% more ‘sweet fruity/floral’ total intensity than basmati and 79% more than Jasmati.

9. Conclusions

2-AP contributes important aroma in many foods, like pandan leaf, mushroom and especially fragrant rice. Amino acids, in particular proline and ornithine, have been identified, alongside reducing sugars, as precursors of 2-AP in both biosynthesis and Maillard reaction. The presence of a non-functional betaine aldehyde dehydrogenase (non-functional BADH2) allows the formation of 2-AP in fragrant rice and several bacteria like *Bacillus cereus* and *Penicillium nalgiovence* may also form 2-AP.

It appears that 1-pyrroline is a key intermediate in both biosynthesis and thermal formation of 2-AP, and this intermediate could form 2-AP through an acylation reaction. 2-Oxopropanal and 2-acetylpyrrolidine are other intermediates hypothesised to form 2-AP during the Maillard reaction and the presence of phosphate ion could increase yields of 2-AP. 2-AP formation mechanisms, particularly in rice, still need to

768 be researched. The work of Poonlaphdecha et al. (2016) showed the importance of 1-
769 pyrroline in 2-AP formation, and future work with this intermediate may provide
770 useful information.

771 Synthesis of 2-AP is still difficult but its stabilisation in a zinc halide complex has
772 increased its applicability. New synthesis strategies and stabilisation techniques could
773 reduce the cost of 2-AP, which may increase its use in the food industry, adding
774 desirable popcorn-like aroma to rice products such as rice cakes. Another possibility
775 is the addition of 2-AP intermediates, such as 1-pyrroline, to rice, which can then
776 readily form 2-AP during processing, providing a desirable fragrance to rice products.

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FIGURE CAPTIONS

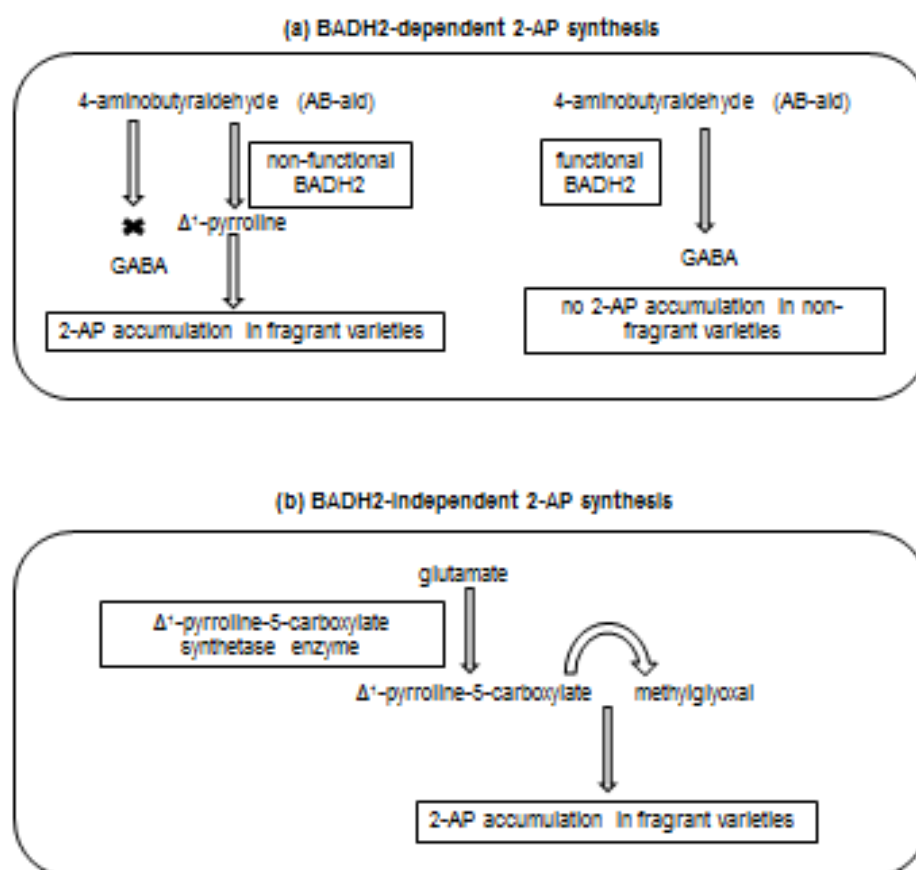
Figure 1. A comparison of the (a) BADH2-dependent 2AP biosynthetic pathway (Bradbury, Gillies, Brusheet, Waters, & Henry, 2008) and the (b) BADH2-independent 2AP biosynthetic pathway (Sakthivel et al., 2009; Huang et al., 2008).

Figure 2. Mechanism of 2-AP formation through the heterolactic pathway (Costello & Henschke, 2002)

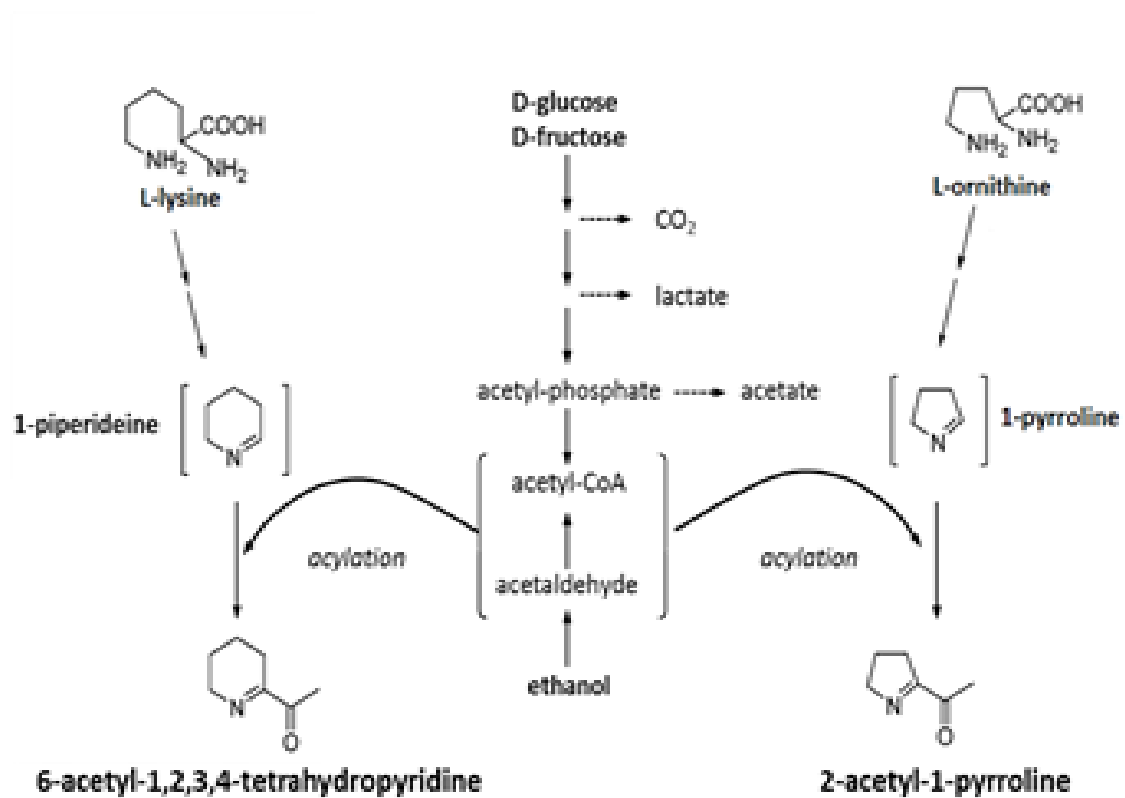
Figure 3. 2-AP Maillard Reaction formation pathways: a) formation of 1-pyrroline from ornithine (Schieberle, 1990); b) formation of 1-pyrroline from proline and 1-deoxyosone (Schieberle, 1995); c) ¹³C-labelled and unlabelled 2-AP formation from 1-pyrroline and ¹³C-glucose (Rewicki et al., 1993).

Figure 4. 2-AP synthesis strategies: a) Buttery, Ling, Juliano, and Turnbaugh, 1983; b) De Kimpe, Stevens, and Keppens, 1993; c. De Kimpe, Stevens, and Keppens, 1993; d) De Kimpe, and Keppens, 1996; e) Favino, Fronza, Fuganti, Fuganti, Grasselli, and Mele, 1996; f) Deblander, Van Aeken, Adams, De Kimpe, & Abbaspour Tehrani (2015).

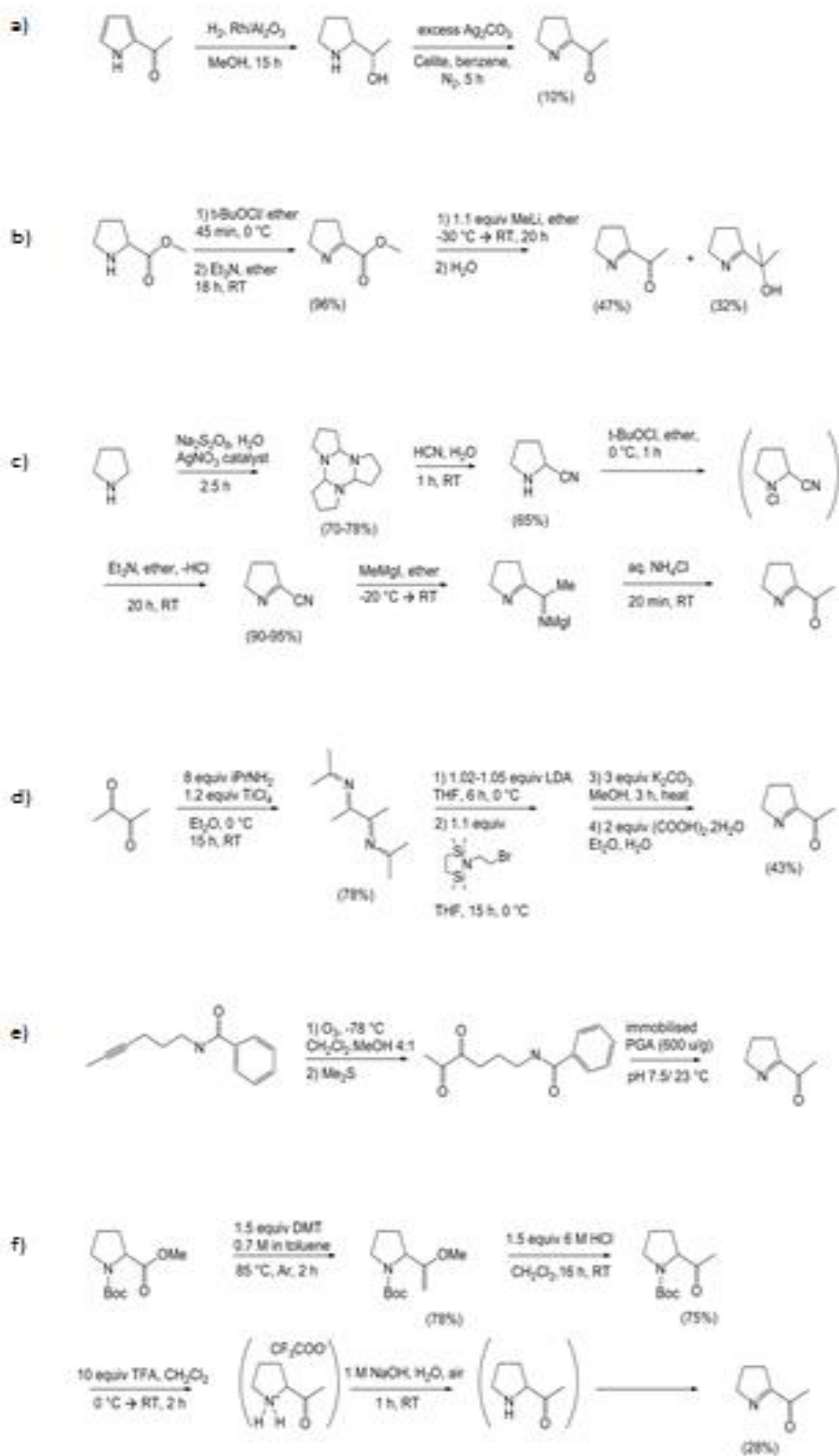
LDA: lithium diisopropylamide; THF: tetrahydrofuran; PGA: immobilised penicillin G acylase; DMT: dimethyltitanocene; TFA: trifluoroacetic acid



1165 Figure 1



1166
1167 Figure 2



1170

1171 Figure 4

Table 1: 2-AP concentrations in fragrant and non-fragrant rice

rice variety	2-AP concentration (µg/kg)	
	milled	brown
<i>fragrant rice</i>		
Basmati	60 ^a 87 ^d 588 ^g 19–342 ^h 434 ^k	170 ^a 610 ^b 119 ^h
Khao Dawk Mali 105	70 ^a 87–532 ⁱ	200 ^a
Malagkit Sungsong	90 ^a	200 ^a 760 ^b
Milagross	70 ^a	
Seratus Malam	60 ^a	
Azucena	40 ^a	160 ^a
Hieri	40 ^a	100 ^a
Ir841-76-1	70 ^a	200 ^a 560 ^b
Jasmine	156 ^d 810 ^h	550 ^h
Della	76 ^d	
Goolarah	691 ^e	
Yrf9	670 ^e	344 ^f
B5-3	2746 ^g	
Amber Aromatic (Lundberg)		345 ^h
Aromatic (Fowler Gourmet)	999 ^h	
Black Thai (Bulk)		259 ^h
Jasmati (Rice Tec)	526 ^h	
Kasmati (Rice Tec)	496 ^h	
Texmati (Rice Tec)	266 ^h	
Aychade	575–638 ^j	
Fidji	45–475 ^j	
Giano	28–336 ^j	
Kala Bhat	920 ^k	
Kali Kumud	732 ^k	
Amritbhog	787 ^k	
<i>non-fragrant rice</i>		
Calrose	<6 ^a	
California Long-Grain	0.6 ^c	
Pelde	15 ^e	

Texas Long Grain	<8 ^a
	6 ^b
Ariette	10.6 ^j
Ruille	24.7 ^j
Sonsali	72 ^k
Kolamb	125 ^k

Data are from the following references: ^aButtery et al., 1983; ^bButtery et al., 1986; ^cButtery et al., 1988; ^dTanchotikul and Hsieh, 1991; ^eWidjaja et al., 1996a; ^fWidjaja et al., 1996b; ^gTava and Bocchi, 1999; ^hBergman et al., 2000; ⁱYoshihashi et al., 2004; ^jMaraval et al., 2010; ^kMathure et al., 2014.

Table 2: 2-AP concentrations in foods other than rice

food sample	2-AP concentration (µg/kg)
wheat bread crusts	75 ^a
Mediterranean dried sausages	750 ^b
bread flowers (<i>Vallaris glabra</i> ktze)	3.36 (fresh) 26.1 (dry) ^c
palm wine	11.4 ^d
roasted Criollo cocoa beans	4.2 ^e
pandan leaves	40–450 ^f
pan-fried mushrooms	4.2–7.0 ^g
roasted in-shell peanuts	1920 ^h
roasted hazelnuts	85 ⁱ
cereal coffee brew	8 ^j
squid broth	97.3 ^k
dark chocolate	21 ^l
milk chocolate	3 ^l
cocoa liquor	11 ^l
Manuka honey	80–450 ^m
raw licorice	9.41 ⁿ
roasted almonds	12 (dry roasted) ^o 30 (oil roasted) ^o

Data are from the following references: ^aSchieberle and Grosch, 1987; ^bStahnke, 2000; ^cWongpornchai et al., 2003; ^dLasekan et al., 2007; ^eFrauendorfer and Schieberle, 2008; ^fYahya et al., 2010; ^gGrosshauser & Schieberle, 2013; ^hKaneko et al., 2013; ⁱKiefl et al., 2013; ^jMajcher et al., 2013; ^kCarrascon et al., 2014; ^lLiu et al., 2015; ^mRuckriemen et al., 2015; ⁿWagber et al., 2016; ^oErten et al., 2017.